Chromosome Evolution in Salamanders of the Genus *Necturus*

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ABSTRACT.— All species of the salamander genus *Necturus* (*N. alabamensis*, *N. beyeri*, *N. lewisi*, *N. maculosus*, and *N. punctatus*) have 19 pairs of chromosomes (2n = 38), and well-differentiated heteromorphic sex chromosomes of the XY male/XX female type. Four distinct karyotypes are observed among the species in terms of C-band patterns, the proportion of asymmetrical chromosomes such as telocentrics, and the degree of differentiation of the sex chromosomes. *Necturus beyeri* appears to be identical to *N. maculosus* in these features while the three other species have uniquely different karyotypes; *N. alabamensis* may be polymorphic for an intermediate number of telocentric chromosomes. Interspecific homologies between the most asymmetrical chromosomes, including telocentrics, are suggested by the similar position of these chromosomes in the karyotypes and similarity in C-band patterns. The species appear to exhibit sequential stages of karyological differentiation in the order: *lewisi-punctatus-alabamensis-beyeri* + *maculosus.* Karyology correlates with geographic distribution in a simple pattern suggesting that gradual karyological differentiation occurred as populations became established southward along the Coastal Plain of southeastern United States, around the southern end of the Appalachian Mountain Range, and into the Mississippi River drainage system. Thus, *N. lewisi* represents the most primitive form and *N. maculosus* the most derived condition.

INTRODUCTION

Salamanders of the genus *Necturus* of eastern North America have been used extensively for biological research for well over 100 years, but the phylogenetic relationship of *Necturus* to other salamanders, and systematics within the genus, remain problematical. *Necturus* represents an ancient lineage of neotenic (*sensu* Gould 1977), permanently aquatic salamanders, with a generalized larval morphology that obscures affinities to other living salamanders (Hecht 1957). Most workers consider *Necturus* to be most closely related to the European blind salamander, *Proteus anguinus*, and place both genera in the same family, Proteidae (Brandon 1969; Larsen and Guthrie 1974; Naylor 1978; Noble 1931;

Morescalchi 1975). Kezer et al. (1965) presented karyological evidence that supports this view. Hecht and Edwards (1976) reviewed the systematic data for these groups of salamanders and concluded that morphological information is not sufficient to resolve this problem, and that more biochemical and karyological studies are needed.

One of the most thorough treatments to date of the genus *Necturus* is that of Hecht (1958), who recognized four species on the bases of external morphology, ontogeny, and geographic distribution: *Necturus beyeri* Viosca, *Necturus lewisi* (Brimley), *Necturus maculosus* (Rafinesque), and *Necturus punctatus* (Gibbes). He also tentatively recognized a subspecies of *N. beyeri*, *N. b. alabamensis* Viosca, but described it as "one of the most distinct forms in the genus" (Hecht 1958:17), and seemed somewhat ambivalent as to its taxonomic status. Subsequently Brode (1970) revised the genus, mainly on osteological grounds, recognizing two species, *N. maculosus* and *N. punctatus*, and six subspecies: *N. m. maculosus*, *N. m. lewisi*, *N. m. walkeri*, *N. p. punctatus*, *N. p. alabamensis*, and *N. p. beyeri*. The present systematic status of this group of salamanders is one of uncertainty and disagreement, mostly because there are so few distinguishing morphological features between the various forms. Nevertheless, most recent workers recognize five species—*N. alabamensis*, *N. beyeri, N. lewisi, N. maculosus,* and *N. punctatus*—as well as several subspecies of *N. maculosus* (Brame 1967; Gorham 1974; Conant 1975).

All species of *Necturus*, with the exception of *N. maculosus,* are distributed along the Coastal Plain of southeastern United States, from southeastern Virginia to eastern Texas (Fig. 1). *Necturus maculosus* is by far the most widely distributed species, with a range extending fan-like from an apex in Louisiana and broadening northward to southeastern Manitoba in the west and southeastern Quebec in the east, essentially encompassing the entire Mississippi River drainage system. The combined ranges of these species result in a more-or-less continuous distribution of *Necturus* over most of eastern North America, interrupted in the east by the Appalachian Mountains which form a wedge separating the two coastal species, *N. lewisi* and *N. punctatus,* from inland populations of *N. maculosus*. According to Brode (1970), partial sympatry and morphological intergraduation may occur between *N. punctatus* and *N. alabamensis,* and between the latter form and *N. beyeri. Necturus lewisi* and *N. punctatus,* however, are the only species definitely known to occur in sympatry, and are morphologically the most distinct of all *Necturus* species (Hecht 1958).

Results of a recent electrophoretic analysis by Ashton et al. (1980) suggest that at least three species, *N. maculosus, N. lewisi,* and *N. punctatus,* are distinct, long-isolated species. Only *N. maculosus* has been studied karyologically (Seto et al. 1964; Morescalchi 1975; Sessions

1980); neither *N. beyeri* nor *N. alabamensis* have been so examined. Relative to other salamanders, *N. maculosus* has a karyotype that is distinctive in chromosome number and morphology, and particularly in the degree of differentiation of the sex chromosomes (Sessions 1980). Our study was carried out to ascertain the degree to which karyological changes have accompanied diversification and divergence within the genus *Necturus*, and to elucidate the relationship between the geographic distribution and the evolutionary history of this group of salamanders.

MATERIALS AND METHODS

The following specimens were used in this study and unless otherwise indicated all have been deposited in the Museum of Vertebrate Zoology, University of California, Berkeley. Abbreviations used below: ASU = Department of Biology, Appalachian State University, Boone, NC; CR = county road; SR = state road.

Necturus alabamensis.— 2♂♂, 1♀, Black Creek at SR 20, 1.6 km W of Bruce, Walton Co., FL; 20 May 1980; 1♀, Juniper Creek, approx. 1.6 km S of Juniper, 137 m elevation, Marion Co., GA; 30 November 1980.

Necturus beyeri.— 1♀, 1♂ (deposited at ASU), approx. 24 km S of Nacogdoches on Bernaldo Creek, Stephen F. Austin State University Experimental Forest, Nacogdoches Co., TX; 29 March 1981.

Necturus lewisi.— 1♂, 1♀, Tar River along State Hwy 44, 2.9 km NNW of Tarboro, Edgecombe Co., NC; January 1980; 1♂, Tar River, 14.1 km S of Nashville, Nash Co., NC; January 1980.

Necturus maculosus.— 1♀, Wisconsin (exact locality not available); from Carolina Biological Supply Co.

Necturus punctatus.— 3♂♂, Little River, crossing of CR 2224, Wake Co., NC; August and January, 1981.

Mitotic chromosomes were prepared from colchicine-treated intestinal epithelium and spleen, following the technique described in Kezer and Sessions (1979). Air and flame dried slides were also made from peripheral blood after *in vivo* treatment with phytohemagglutinin (PHA, Sigma) and Colcemid (Sigma). C-banding was carried out on squash preparations of intestinal epithelium and spleen cells, using the technique of Schmid et al. (1979). At least three mitotic cells were examined from each individual. Idiograms were constructed from measurements of a single representative mitotic spread for *N. alabamensis, N. lewisi, N. maculosus,* and *N. punctatus.*

RESULTS

All species of *Necturus* studied have 19 pairs of chromosomes (2n = 38), including a pair of well-differentiated heteromorphic sex chromosomes of the XY male/XX female type. In terms of chromosome morphology and degree of differentiation of the X and Y sex chromosomes, we discern at least four unique karyotypes in the genus (Fig. 2a-d). These are represented by idiograms in Figure 3.

Necturus lewisi has a completely bi-armed karyotype (Figs. 2a, 3; Table 1), and the X-chromosome is a medium-size metacentric so that the sex chromosome pair is in position 6 of the karyotype. The Y-chromosome of this species is subtelocentric and mostly euchromatic (unstained) after C-banding, except for two bands of heterochromatin, one light and one dark, in the middle of the long arm, and the proximal portion of the long arm which is lightly stained (Fig. 4). Two chromosomes (besides the Y-chromosome) are extremely asymmetrical (subtelocentric) with large arm ratios (Fig. 3; Table 1).

Necturus punctatus also lacks telocentric chromosomes (Figs. 2b, 3; Table 1), but its X-chromosome is larger than that of *N. lewisis* and the sex chromosomes are in position 3 or 4 of the karyotype. *Necturus punctatus* has three pairs of subtelocentric chromosomes (besides the Y-chromosome), two medium-size and one small, with large arm ratios (Table 1). The medium-size subtelocentrics of *N. punctatus* are in similar positions in the karyotype to the most asymmetrical chromosomes of *N. lewisi* (Fig. 3). The subtelocentric Y-chromosome of *N. punctatus* is considerably more heterochromatic than the Y-chromosome of *N. lewisi*, but less heterochromatic than the Y-chromosome of *N. maculosus* (Fig. 4).

We have found at least two and possibly three different karyomorphs in specimens collected from the range of *N. alabamensis*, which differ in the number of telocentric chromosomes. The karyotype of a female from Georgia is identical to that of *N. maculosus*, with 12 (6 pairs) telocentric chromosomes. A male and female from Florida, on the other hand, have karyotypes with only 8 (4 pairs) telocentric chromosomes (Fig. 2c). A third specimen from the same locality in Florida may have 10 (5 pairs) telocentrics, although this count was based on three mitotic preparations of mediocre quality. A male specimen with eight telocentrics was used for the construction of an idiogram (Fig. 3). In this karyotype, two of the telocentric chromosomes are medium-size and two are small (Table 1; Fig. 3). The medium-size telocentrics are in positions in the karyotype similar to the most asymmetrical medium-size chromosomes in *N. lewisi* and *N. punctatus*, suggesting interspecific homology of these chromosomes (Fig. 3). The sex chromosomes of this specimen fall into position 3 of the karyotype (Fig. 3), as in *N. maculosus* (Sessions 1980; this paper). In addition, the subtelocentric Y-chromosome is more heterochromatic with a more complex banding pattern than in *N. punctatus*, but is somewhat less heterochromatic than the Y-chromosome of *N. maculosus* (Fig. 4).

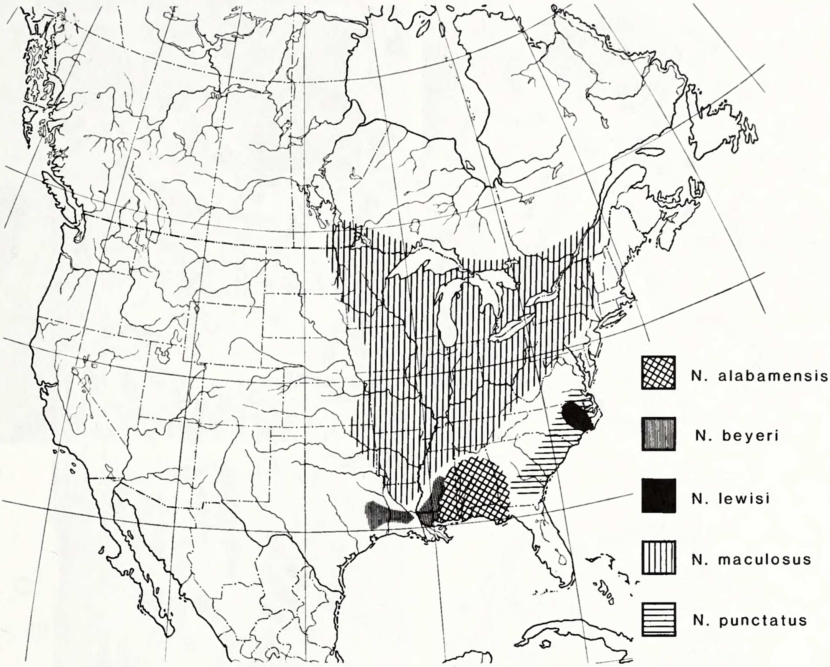

Fig. 1. Map showing approximate distribution of species of the genus *Necturus* used in this study (adapted from Conant 1975).

Specimens of *N. beyeri* from Texas have identical karyotypes to that of *N. maculosus*, with 12 (6 pairs) telocentric chromosomes (Fig. 5). As in the other species, two of these pairs of asymmetrical chromosomes are medium-size, and the remaining telocentrics are small. The Y-chromosome of *N. beyeri* is also virtually identical to that of *N. maculosus*.

A karyotype and idiogram of *N. maculosus* were presented earlier (Sessions 1980), but a new C-band idiogram of this species was constructed for this study (Fig. 3). It shows the position of the sex chromosomes and the 6 pairs of telocentric chromosomes in the karyotype. The subtelocentric Y-chromosome of this species is more heterochromatic than that in any of the other species of *Necturus* (Fig. 4).

DISCUSSION

Our karyological data support previously reported electrophoretic evidence (Ashton et al. 1980) that *N. lewisi, N. punctatus,* and *N. maculosus* are distinct, probably long-isolated species. These data further suggest that *N. alabamensis*, which has not been studied electrophoretically, may represent another distinct species, and that *N. beyeri* is only a little-differentiated southern form of *N. maculosus.* Karyological evi-

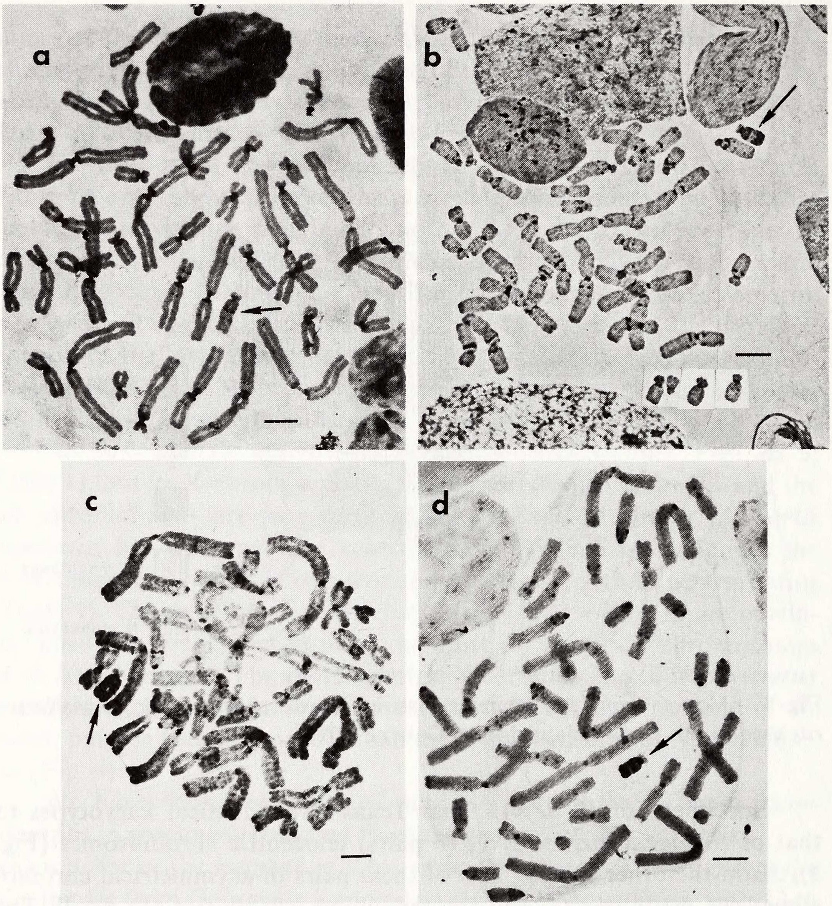

Fig. 2a-d. C-banded mitotic preparations of *Necturus* species: a, *N. lewisi*; b, *N. punctatus*; c, *N. alabamensis* (8 telocentrics); d, *N. maculosus*. Arrows indicate Y-chromosomes. Scale = 10 micrometers.

dence is also in accord with electrophoretic data in that *N. lewisi* and *N. punctatus* are more similar to each other than either is to *N. maculosus*. The karyological information, however, reveals additional details of interrelationships that have not been resolvable by the electrophoretic analysis.

While all species of *Necturus* have the same diploid number of chromosomes, they differ in the degree of karyotype asymmetry (Table 2). The species form two groups on the basis of presence or absence of telocentric chromosomes: *N. lewisi* and *N. punctatus* have completely bi-armed karyotypes, while *N. maculosus, N. beyeri,* and *N. alabamensis* all have several pairs of telocentric chromosomes. In terms of

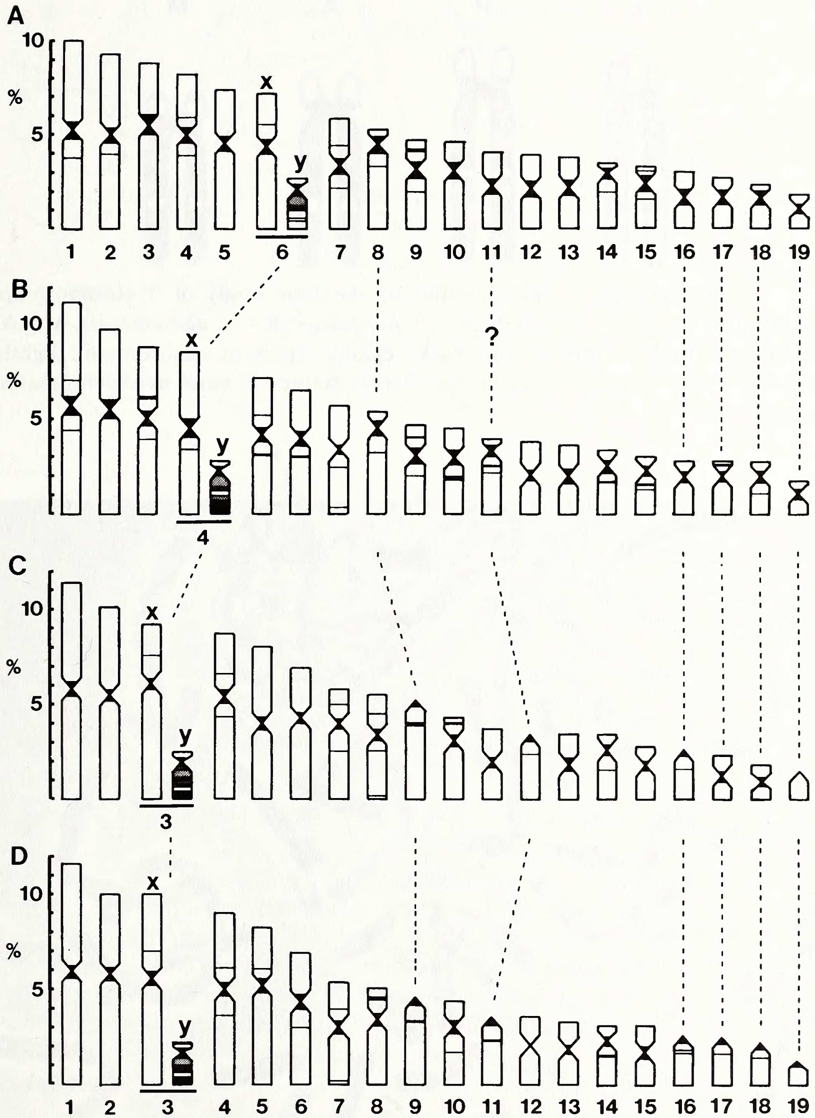

Fig. 3. Idiograms of *Necturus* species showing four kinds of karyotypes found in the genus. A, *N. lewisi*; B, *N. punctatus*; C. *N. alabamensis*; D, *N. maculosus*. Dark regions and lines represent C-band heterochromatin. Stippled areas on Y-chromosomes represent light staining C-band heterochromatin. Presumed homologous chromosomes are connected by dashed lines between idiograms.

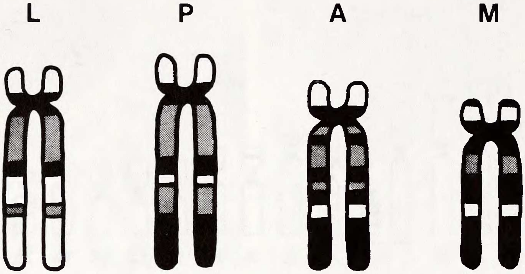

Fig. 4. Diagrammatic representation of the four kinds of Y-chromosomes found in *Necturus*. L = *N. lewisi*, P = *N. punctatus*, A = *N. alabamensis*, M = *N. maculosus*. Light, stippled, and dark regions represent euchromatin, lightly stained C-band heterochromatin, and darkly stained C-band heterochromatin, respectively.

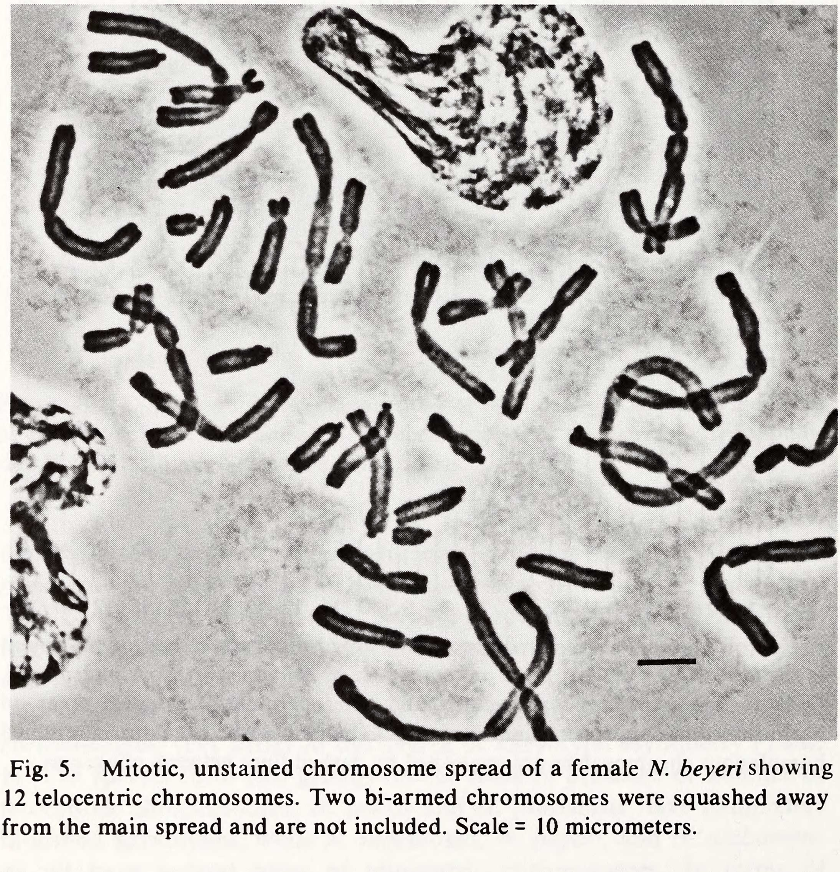

Fig. 5. Mitotic, unstained chromosome spread of a female *N. beyeri* showing 12 telocentric chromosomes. Two bi-armed chromosomes were squashed away from the main spread and are not included. Scale = 10 micrometers.

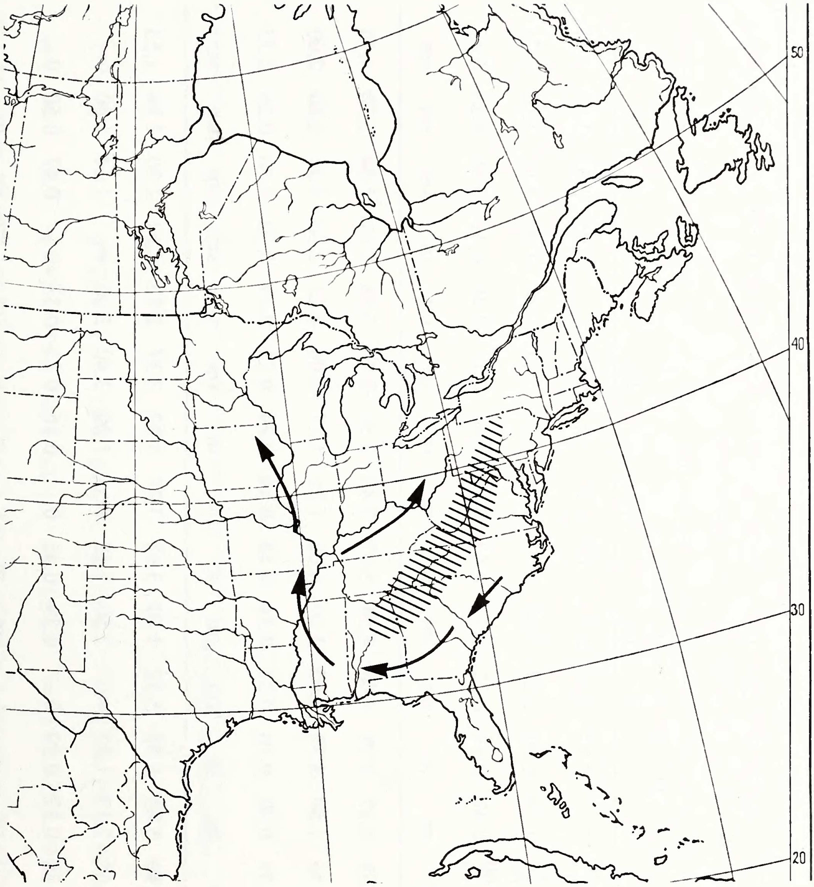

Fig. 6. Map showing relationship between geographic distribution and phylogenetic history in the genus *Necturus*. Karyological differentiation occurred as populations of *Necturus* become established increasingly southward along the coastal plain east and south of the Appalachian Mountains (designated by hatching). Eventual arrival at the Mississippi River drainage system allowed explosive northward dispersal of the karyologically most derived form, *N. maculosus*.

chromosome asymmetry, sex chromosome differentiation, and/or proportion of telocentric chromosomes, *N. punctatus* and *N. alabamensis* have seemingly intermediate karyotypes. Since these two species also have a somewhat intermediate geographic distribution, we hypothesize that karyological evolution within the genus involved progressive differentiation of the X and Y sex chromosomes, through increasing heterochromatinization of the Y-chromosome and increasing relative size of the X-chromosome, with a concomitant increase in the degree of chromosomal asymmetry through pericentric inversion or some other

Table 1. Analysis of chromosome complements of *Necturus lewisi* (*L*), *N. punctatus* (*P*), *N. alabamensis* (*A*), and *N. maculosus* (*M*). RL = relative length (% of total genome). AR = arm ratio (long arm/short arm). CI = centromere index (short arm/total chromosome length). CP = centromere position, terminology after Levan et al. (1964) and Green et al. (1980); m = metacentric, sm = submetacentric, t = telocentric, st = subtelocentric.

		Chromosome number																		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
L	RL	10.3	9.33	8.76	8.19	7.43	7.24[a] 2.67	5.91	5.33	4.67	4.57	4.00	3.91	3.81	3.52	3.33	2.95	2.67	2.29	1.81
	AR	1.08	1.13	1.71	1.53	1.60	1.53 4.60	1.39	6.00	2.27	2.20	1.33	1.28	1.50	5.17	2.89	1.39	1.80	3.00	1.71
	CI	0.48	0.46	0.36	0.40	0.39	0.40 0.18	0.42	0.14	0.31	0.31	0.43	0.44	0.40	0.16	0.26	0.42	0.36	0.25	0.37
	CP	m	m	sm	m	m	m st	m	st	sm	sm	m	m	m	st	sm	m	sm	sm	sm
P	RL	11.1	9.65	8.79	8.51[a] 2.84	7.09	6.53	5.67	5.39	4.68	4.54	3.97	3.83	3.55	3.40	2.98	2.84	2.84	2.70	1.70
	AR	1.05	1.35	1.30	1.14 4.00	1.38	1.56	1.50	5.33	2.00	2.20	6.00	1.25	1.27	3.00	4.25	2.33	2.33	2.80	2.00
	CI	0.49	0.43	0.44	0.47 0.20	0.42	0.39	0.40	0.16	0.33	0.31	0.14	0.44	0.44	0.25	0.19	0.30	0.30	0.26	0.33
	CP	m	m	m	m st	m	m	m	st	sm	sm	st	m	m	sm	st	sm	sm	sm	sm
A	RL	11.4	10.1	9.20[a] 2.45	8.74	7.98	6.90	5.83	5.52	5.22	4.30	3.68	3.37	3.37	3.37	2.76	2.61	2.30	1.84	1.53
	AR	1.06	1.20	2.00 4.33	1.59	1.00	1.65	2.17	1.57	∞	2.50	1.18	∞	1.20	3.40	2.00	∞	1.14	1.00	∞
	CI	0.48	0.45	0.33 0.19	0.39	0.50	0.38	0.32	0.39	0	0.29	0.46	0	0.46	0.23	0.33	0	0.47	0.50	0
	CP	m	m	sm st	m	m	m	sm	m	t	sm	m	t	m	st	sm	t	m	m	t

M	RL	11.6	9.98	9.98[a] 2.20	8.98	8.19	6.89	5.29	5.09	4.55	4.35	3.59	3.55	3.29	3.09	3.09	2.60	2.50	2.20	1.20
	AR	1.05	1.38	1.27 4.50	1.25	1.73	1.76	1.41	2.19	∞	2.35	∞	1.35	1.36	3.43	1.21	∞	∞	∞	∞
	CI	0.49	0.42	0.44 0.18	0.44	0.37	0.36	0.42	0.31	0	0.30	0	0.44	0.43	0.23	0.45	0	0	0	0
	CP	m	m	m st	m	sm	sm	m	sm	t	sm	t	m	m	st	m	t	t	t	t

[a] heteromorphic pair: X-chromosome top, Y-chromosome bottom.

Table 2. Summary of chromosome morphology of four species of *Necturus*, from Table 1 (disregarding subtelocentric Y-chromosomes).

	Chromosome morphology				
Species	m	sm	st	t	% asymmetric chromosomes (t + st)
lewisi	10	7	2	0	10.5
punctatus	9	7	3	0	15.5
alabamensis	10	4	1	4	26.3
maculosus	8	4	1	6	36.8

kind of non-Robertsonian mechanism of centromere shifts. This latter process may represent an example of the phenomenon referred to by White (1973) as "karyotypic orthoselection," though it is not clear how selection could be involved in this case.

We consider the karyotype of *N. lewisi* to represent the primitive condition within the genus, since this species has the least differentiated sex chromosomes. *Necturus lewisi* has the most restricted range of any *Necturus* and may represent a relict.

Necturus punctatus has more differentiated sex chromosomes than *N. lewisi*; the X-chromosome is larger relative to the Y-chromosome, and the Y-chromosome is more heterochromatic. In this species, all chromosomes are bi-armed, but they show more asymmetry in centromere position than is seen in *N. lewisi* (Table 2). The similar sizes and C-band patterns of the most asymmetrical chromosomes in both species suggest that they are homologous chromosomes (Fig. 3). The geographic distribution of *N. punctatus* includes that of *N. lewisi,* but is much larger, extending southward to possibly overlap with the range of *N. alabamensis* in the southern Gulf states (Fig. 1).

Necturus alabamensis has more highly differentiated sex chromosomes than *N. punctatus*, in terms of Y-chromosome heterochromatin, and an even more asymmetrical karyotype with at least four pairs of telocentric chromosomes (Table 2; Fig. 3). This species is found south of the southern limit of the Appalachian mountain range and, with *N. beyeri*, is located at the southern end of the range of *N. maculosus* (Fig. 1). *Necturus beyeri* appears to be identical to *N. maculosus* in number of telocentrics and in Y-chromosome differentiation.

At least two and possibly three karyotypes were encountered in specimens collected in the geographic range of *N. alabamensis*, with 8, 10, and 12 telocentrics. Several interpretations of this apparent karyological variability are possible. Perhaps *N. alabamensis* represents a karyological intergrade between *N. punctatus* and *N. beyeri*. If this is so, then heterozygotes with heteromorphic telocentric/bi-armed chromosome pairs should occur. Such heterozygotes, however, were not found in this investigation. Furthermore, the Y-chromosome of the 8-telocentric male *N. alabamensis* differs in its C-band pattern from both *N. punctatus* and *N. beyeri*, suggesting that it is a distinct form. Although C-band information is not available for the 10- and 12-telocentric specimens collected in the range of *N. alabamensis*, our tentative conclusion is that *N. alabamensis* is a chromosomally polymorphic species, possibly exhibiting clinal variation in number of telocentrics. Resolution of this probably complex problem awaits more extensive karyological and biochemical investigations.

Necturus maculosus may be the only totally allopatric species of *Necturus*, except in regions where it may have been recently introduced (Ashton et al. 1980), and has by far the largest range of any of the species. Yet, virtually no molecular divergence is detectable between populations of this species (Ashton et al. 1980). Specimens of *N. punctatus* taken from two different river systems less than 200 km apart in North Carolina showed more genetic divergence from each other than did specimens of *N. maculosus* collected from widely disparate localities in Wisconsin, Massachusetts, and North Carolina (Ashton et al 1980). The electrophoretic and karyological patterns observed probably reflect the streambound life style of these neotenic salamanders, which represent a lineage that may have been permanently aquatic since the Paleocene (Naylor 1978). The isolation of populations of *Necturus* in parallel river systems imposes a constraint on dispersal patterns, and has probably encouraged *in situ* chromosomal and genetic differentiation. The peculiar geographic distribution and pattern of genetic differentiation of *N. maculosus* relative to its congeners is probably due to its occupancy of the vast, highly branched, north-south flowing Mississippi River system.

The differences observed in the heteromorphic sex chromosomes among the species of *Necturus* may reflect evolutionary differentiation of these elements in a manner similar to that hypothesized by Ohno (1967). If so, then the sex chromosomes of *Necturus* species can be used, in conjunction with electrophoretic and distributional data, to reconstruct certain aspects of the phylogenetic history of *Necturus* in North America.

From a karyological viewpoint, the geographic distribution of *Necturus* species can be interpreted as a "karyomorphocline", with the southeast coastal species showing increasing karyological differentiation southward and then westward along the Gulf coast, and finally into the Mississippi River drainage system (Fig. 6). The Appalachian Mountains form a natural barrier to westward dispersal of the northern coastal populations and to eastward dispersal of *N. maculosus*. A somewhat analogous situation exists in subspecies of the pickerel, *Esox americanus* (Crossman 1966). In contrast to our interpretation of the *Necturus* pattern, two subspecies of pickerels are thought to have spread northward, one on each side of the Appalachians from a common origin at the southern end, with secondary contact at the southern end producing intergrades (Crossman 1966). Of relevance to the present distribution of *Necturus* species as well as *Esox americanus*, however, is the contrast between the relatively short, eastward and parallel flowing river systems on the east side of the Appalachians and the vast Mississippi River system flowing southward from Canada to Louisiana on the west side: north-south spreading of such stream-bound, permanently aquatic organisms was probably very slow along the Atlantic coast relative to south-north spreading in the inland area.

Presumably, karyological differentiation in *Necturus*, involving increasing chromosomal asymmetry and progressive changes in sex chromosome morphology, gradually occurred as populations became established farther south, around the southern end of the Appalachians, and into the Mississippi River drainage system. *Necturus maculosus* (including the southern form, *N. beyeri*) is the culmination of these karyological and geographic trends and represents the most derived state. It has an extensive, fanlike distribution and probably represents one vast, genetically and karyologically homogeneous population. The distribution of *N. maculosus* was probably the result of a relatively recent and explosive northward dispersal of this species in response to access to the extensive Mississippi River drainage system. This hypothesis awaits substantiation by further electrophoretic, karyological, and ecological studies.

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